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Protein Expression and Purification 35 (2004) 1–10
Protein Expression & Purification
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Refolding and purification of unprocessed porcine myostatin expressed in *Escherichia coli*

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Received 10 June 2003, and in revised form 11 December 2003

Abstract

Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily, and it acts as a negative regulator for skeletal muscle growth. Like many other TGF-β family member proteins, the mature form of myostatin is a homodimer that is processed post-translationally from a precursor form of myostatin. Since the presence of a prodomain is essential for proper folding and homodimer assembly for some members of the TGF-β superfamily, we compared the refolding in vitro of porcine unprocessed and mature myostatin over-expressed in *Escherichia coli* as inclusion bodies. A high alkaline buffer solution containing a mild anionic detergent and a reducing agent was used to solubilize the myostatin inclusion bodies. An optimal condition for refolding was obtained by rapid dilution of the solubilized protein in a buffer system containing reduced and oxidized glutathione, and subsequent incubation at 4 °C for at least 7 days. The unprocessed porcine myostatin demonstrated reversible disulfide bond formation after refolding, a characteristic of the native form of myostatin. In contrast, the mature myostatin formed aggregates that did not demonstrate reversible disulfide bond formation in the refolding condition used in this study. These results demonstrate the importance of the myostatin prodomain in facilitating the proper folding of mature myostatin. Reaction of the refolded, unprocessed myostatin with furin, an endopeptidase cleaving between paired basic residues, yielded prodomain and mature myostatin, demonstrating that the unprocessed myostatin is a substrate for furin. The prodomain did not form disulfide bond formation but the mature myostatin demonstrated reversible disulfide-linked homodimer formation. It is concluded that myostatin prodomain facilitates the proper folding of myostatin, and the refolded, native form of unprocessed myostatin could be obtained in high yield (15%) after *E. coli* expression as inclusion bodies.

Keywords: Refolding; Purification; Myostatin

Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily, which is expressed almost exclusively in skeletal muscle and acts as a negative regulator for skeletal muscle growth. Myostatin knockout mice exhibited a 2–3-fold increase in skeletal muscle mass without any impact on the size of other organs as compared to wild-type mice [1]. Dysfunctional mutations in the myostatin gene have been observed in 'double muscled' cattle [2–4]. In addition, systemic administration of myostatin induced significant muscle wasting in mice [5]. Conversely, administration of anti-myostatin antibody to adult mice significantly increased skeletal muscle mass [6].

Like many other TGF-β family member proteins, myostatin appears to be produced as a precursor protein composed of a signal sequence, a N-terminal propeptide domain (prodomain), and a C-terminal mature (active) domain [5]. Based on its cDNA sequence, the precursor form of myostatin is known to comprise 375 amino acids in humans, baboons, cattle, pigs, sheep, turkeys, and chickens, and 376 amino acids in rodents [2]. The mature form of myostatin, consisting of 109 amino acids, appears to be formed upon removal of the N-terminal prodomain by proteolytic cleavage at the tetrabasic

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doi:10.1016/j.pep.2004.01.001

(Arg–Lys–Arg–Arg) site [7]. The amino acid sequence of the mature form of myostatin is identical among human, murine, rat, porcine, chicken, and turkey species, with only a few amino acid differences in other mammalian species, exhibiting a remarkable conservation through evolution [2]. Various studies indicate that the mature form of myostatin, as well as the precursor form of myostatin, forms a disulfide-linked dimer like many other members of the TGF-β superfamily [1,7,8]. Furthermore, it was demonstrated that the myostatin prodomain binds non-covalently with the mature myostatin to make a latent form, resulting in inhibition of the biological activity of myostatin [8].

Easy availability of large quantities of myostatin will be useful in investigating the physiological role of myostatin in vivo. While refolding is often a major drawback in *E. coli* expression of recombinant proteins, *E. coli* expression allows economic production of large amounts of recombinant protein. A few studies have reported the production of recombinant, mature myostatin in *E. coli* systems [9,10], but details on the yield, refolding, and biochemical characteristics of the refolded myostatin are lacking. Besides, it has been demonstrated that the presence of the prodomain is essential for proper folding and homodimer assembly in some members of the TGF-β superfamily such as activin A, TGF-β1, and bone morphogenetic protein-2 [11,12] as well as in a host of other proteins [13,14]. In this study, we compared the refolding between the unprocessed porcine myostatin and mature myostatin expressed in *E. coli*. The refolding results indicate that the prodomain of myostatin aids refolding in vitro. We also present data on purification and biochemical characterization of the refolded, unprocessed myostatin.

Materials and methods

Amplification of porcine myostatin cDNA

Total RNA was extracted from a pig *longissimus dorsi* muscle using a commercial extraction kit (Trizol, Gibco-BRL, Rockville, MD) and mRNA was purified from the total RNA using an oligo(dT) column. Messenger RNAs (100 ng) were subjected to oligo-dT primed reverse transcription using 200 U SuperScript II RNase H^- reverse transcriptase (Gibco-BRL, Rockville, MD) to synthesize cDNAs. Five μl of the above reverse transcription reaction mixture was used to amplify 1065 bp unprocessed and 369 bp C-terminal fragment porcine myostatin in a total volume of 50 μl with 1 U *Taq* polymerase mixture (PCR Supermix High Fidelity, Gibco-BRL, Rockville, MD) and 0.2 μM primers with the following parameters: 2 min initial denaturation at 94 °C; 30 s denaturation at 94 °C, 30 s annealing at 58 °C, 2 min extension at 72 °C (30 cycles), and 5 min final extension at 72 °C. Primers were designed on the basis of the reported porcine myostatin mRNA sequence (GenBank Accession No. AF019623). The forward primers for the 1065 bp unprocessed myostatin and 369 bp mature myostatin were 5′-GTGGATCTGAATGAGAAC AGCGAGC -3′ and 5′-GAGGTCAGAGTTACAGAC ACA-3′, respectively, and the reverse primer for both fragments was 5′-TCATGAGCACCCACAGCGATC T-3′. The primer combination for the unprocessed and mature myostatin was designed to yield PCR products corresponding to bases 61–1125 and 760–1125 of porcine myostatin mRNA sequences, respectively.

Construction of myostatin expression vector

Amplified myostatin cDNAs were purified from PCR mixtures using a commercially available PCR purification kit (Amersham–Pharmacia, Piscataway, NJ) and subsequently inserted into a cloning vector (pCR T7/NT-TOPO, Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Recombinant DNAs were introduced by heat shock into competent *E. coli* for characterization of construct, propagation, and maintenance. Clones were identified for correct insertion using PCR and restriction analysis of plasmids carrying the recombinant cDNAs. After the initial screening, plasmids of selective clones were subjected to DNA sequence analysis for the final selection of an expression plasmid carrying myostatin sequence that was 100% homologous to the reported sequence.

Expression of recombinant myostatin

The plasmid carrying the 100% homologous myostatin sequence was isolated and introduced by heat shock into expression competent *E. coli* harboring the lambda DE3 lysogen that carries the T7 RNA polymerase under the control of the *lac* UV5 promoter [15]. The transformation mixture (250 μl) was added to LB medium (10 ml) containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for overnight growth at 37 °C with vigorous shaking. This culture was used to inoculate 200 ml of prewarmed LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The expression of the recombinant myostatins was induced at OD_{600} of 0.6–0.7 by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM and grown for 4 h. After induction, cells were harvested by centrifugation at 5000*g* for 20 min and stored frozen for later use.

Isolation of inclusion bodies containing recombinant myostatin

For isolation of inclusion bodies containing myostatin, cells were lysed by the combination of sonication and three cycles of flash freeze in liquid nitrogen and

thaw at 37 °C. The insoluble materials were collected by centrifugation at 5000*g* for 20 min and washed twice in a buffer containing 50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, and 10 mM imidazole (pH 7.8). The pellet was washed twice again with distilled water to remove salts and detergents. The final sediment containing inclusion bodies was used for solubilization.

Solubilization and refolding of recombinant myostatin

Inclusion bodies were solubilized (1 mg/ml) in a commercially (Novagen, Madison, WI) available buffer solution (50 mM CAPS, pH 11.0, 0.3% N-laurylsarcosin, and 1 mM dithiothreitol) for 15 min at room temperature and centrifuged at 10,000*g* for 10 min to remove cell debris and insoluble proteins. The supernatant containing the solubilized myostatin was rapidly diluted with cold refolding buffer (20 mM Tris–HCl, pH 8.5, 0.15 M NaCl, 1 mM DTT, 1.3 mM reduced glutathione, and 1.0 mM oxidized glutathione) to a final concentration of 10 μg/ml, and then vortexed vigrously for 1 min at room temperature before incubation at 4 °C for 1 week for refolding. After 1 week of refolding, the refolding mixture was dialyzed overnight in a 20 mM Tris–HCl buffer (pH 8.5) at 4 °C with two changes of buffer during dialysis.

Anion-exchange chromatography of refolded myostatin

After filtration through a 0.22 μm filter, 150 ml (1.5 mg) of the refolded myostatin solution was applied to an anion exchange column (Hiprep 10/16 DEAE, Amersham–Pharmacia, Piscataway, NJ) equilibrated with 20 mM Tris–HCl (pH 8.5) to purify the refolded myostatin. After loading, the column was washed with equilibration buffer, and elution was carried out with a linear gradient of Tris–HCl (pH 8.5) from 20 mM to 1 M over 60 min at a flow rate of 4 ml/min. The absorbance was monitored at 280 nm and 2 ml fractions were collected during elution. Myostatin containing peaks were identified by SDS–PAGE analysis.

Affinity chromatography of refolded myostatin

Since the recombinant myostatin contained a N-terminal His_6 tag as a fusion protein, affinity chromatography was also used to purify refolded myostatin. After filtration through a 0.22 μm filter, the refolded myostatin solution (250 ml) was loaded into an immobilized nickel affinity column (HisBind Quick Column, Novagen, Madison, WI) equilibrated with 20 mM Tris–HCl (pH 8.5) containing 0.5 M NaCl. After loading, the column was washed with equilibration buffer and then eluted stepwise with equilibration buffer containing 20 and 50 mM EDTA at a flow rate of 1.74 ml/min. The absorbance was monitored at 280 nm and 2 ml fractions were collected.

Size-exclusion gel chromatography

Refolded myostatin purified by either ion-exchange or affinity chromatography was subjected to size-exclusion gel chromatography in a FPLC system. About 11 μg of refolded myostatin was applied to a Superdex 200 HR 10/30 column (Amersham–Pharmacia, Piscataway, NJ) equilibrated with 20 mM Tris–HCl (pH 8.5) containing 150 mM NaCl and eluted with the same buffer at a flow rate of 0.5 ml/min. The absorbance was monitored at 280 nm and 0.5 ml fractions were collected.

Isoelectro-focusing

To estimate the isoelectric point and homogeneity of the refolded myostatin, isoelectro-focusing (IEF) was conducted using a 4 × 5 cm commercial IEF gel (Bio-Rad, Hercules, CA) with a pH gradient from 4.45 to 9.6 following the manufacturer's protocol. Anode buffer was 7 mM phosphoric acid and cathode buffer was 20 mM lysine and 20 mM arginine. After fractionation, gels were fixed with trichloroacetic solution as described by Robertson et al. [16] and then stained with Coomassie brilliant blue solution.

Native page

Protein samples (3.4 μg) were mixed with commercial loading buffer (Bio-Rad, Hercules, CA), loaded into a 10% polyacrylamide gel without SDS, and then fractionated in a buffer solution (25 mM Tris–HCl, 192 mM glycine, pH 8.8) containing no SDS. Protein bands were stained with Coomassie brilliant blue solution.

SDS-page

SDS–PAGE was performed by the method of Laemmli [17]. Samples were mixed with loading buffer in both the presence and absence of 1.5% β-mercaptoethanol and boiled for 5 min before loading. After electrophoresis, bands were visualized by Coomassie brilliant blue or silver staining.

Furin proteolysis of refolded myostatin

Furin, an endopeptidase recognizing the paired basic residues (Arg–Lys–Arg–Arg), was purchased from New England BioLabs (Beverly, MA). 3.4 μg of purified refolded myostatin was mixed with 6 U of furin in 15 μl reaction buffer (0.1 M Hepes, pH 7.5, 0.5% Triton X-100, 1 mM $CaCl_2$, and 1 mM β-mercaptoethanol),

and the reaction was incubated at 30 °C overnight. The reaction products were analyzed by SDS–PAGE.

Western blotting

Western blotting was performed to determine whether the refolded myostatin had an affinity to a commercially available anti-myostatin antibody raised against a synthetic peptide from the amino acid sequence of mature myostatin (Santa Cruz Biotechnology, Santa Cruz, CA). Myostatins fractionated by 15% SDS–PAGE were electrophoretically transferred to a nitrocellulose membrane. After blocking with TBST (100 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20), the membrane was incubated with polyclonal anti-myostatin antibody at 1:200 dilution for 1 h at room temperature. After washing with TBST, the membrane was incubated with a 1:5000 dilution of rabbit anti-goat IgG antibodies conjugated with alkaline phosphatase for 1 h at room temperature, followed by color development with the BCIP/NBT substrate (Sigma, St. Louis, MO).

Results

Expression of recombinant porcine myostatin and inclusion body preparation

The expression of unprocessed and mature myostatins is shown in Fig. 1A. Typically, a 4 h incubation was enough to induce maximum expression after IPTG addition. The expressed recombinant myostatins were designed to consist of a N-terminal 3.9 kDa fusion protein originating from the cloning vector and either a 40.4 kDa unprocessed and a 14 kDa mature myostatin sequence, thus the estimated molecular weights of the expressed unprocessed and mature myostatins were around 44.3 and 17.9 kDa, respectively. On SDS–PAGE under reduced conditions, the recombinant myostatins migrated as a band at about 50 and 20 kDa (Fig. 1). The recombinant myostatins were expressed as inclusion bodies, and as shown in Fig. 1B, the isolated myostatin inclusion bodies were relatively pure without many contaminating proteins. The purified inclusion bodies were used for subsequent solubilization and refolding. About 16 mg of inclusion body proteins was harvested for both the unprocessed and mature myostatins from 200 ml culture.

Refolding of myostatin solubilized from inclusion bodies

To determine the appropriate incubation time for refolding, samples were collected periodically (6 h, 1, 3, 5, 7, and 10 days) during the incubation of solubilized inclusion bodies in refolding buffer. The SDS–PAGE analysis of the refolding samples collected during various incubation periods is shown in Fig. 2. Since myostatin is known to form a disulfide dimer in its non-reduced native state [1,7,8], the appearance of myostatin dimers under non-reduced SDS–PAGE analysis was used as an indication of the proper refolding of the recombinant myostatin. As shown in Fig. 2A, a 100 kDa unprocessed myostatin dimer band (upper arrow) as

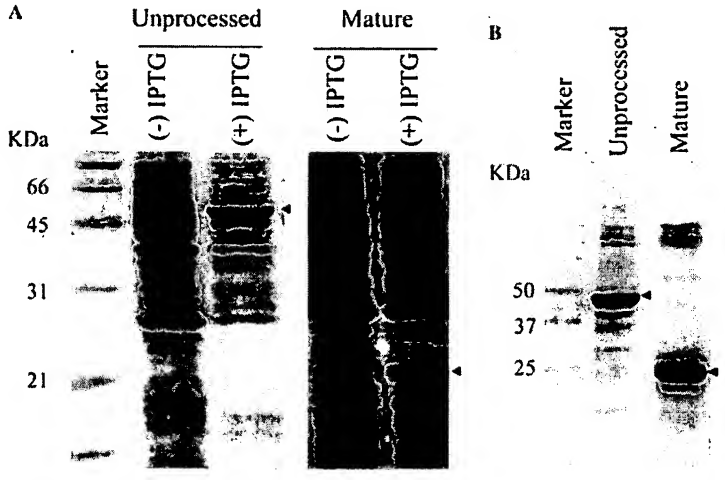

Fig. 1. SDS–PAGE analysis of the expression of recombinant myostatins and myostatin inclusion body preparation. (A) Transformed *E. coli* cells were grown in LB medium until the OD_{600} of the culture reached about 0.5. Expression was induced by adding IPTG, then culture samples were collected during fermentation for the confirmation of expression. Proteins in cell pellets were fractionated by 18% reduced SDS–PAGE and protein bands were visualized by Coomassie blue staining. (B) Inclusion bodies prepared from cell pellets were subjected to reduced SDS–PAGE analysis and visualized by Coomassie blue staining. Upper and lower arrowheads indicate the unprocessed and mature myostatins, respectively. Molecular weight markers are indicated in kDa.

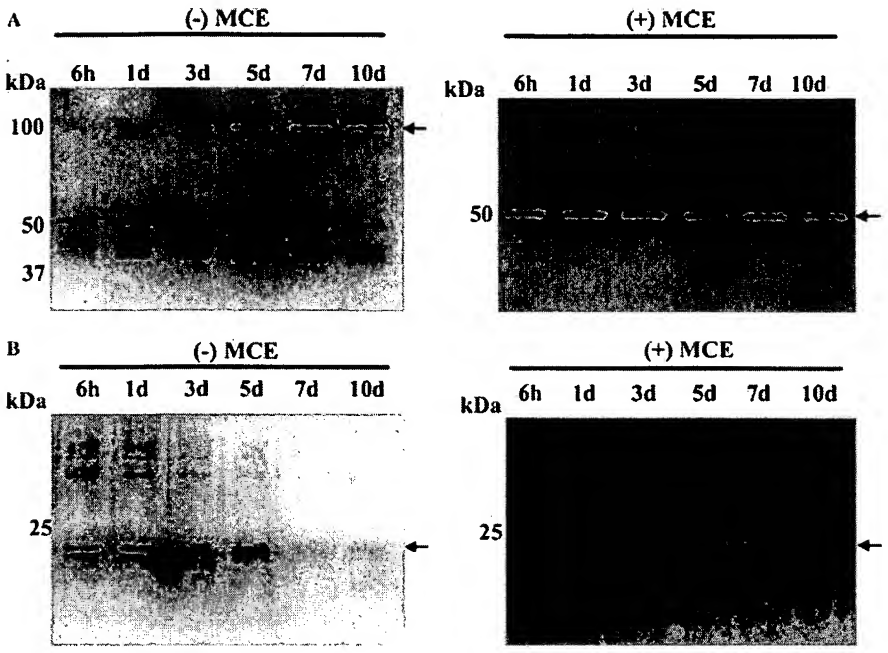

Fig. 2. Refolding of solubilized recombinant myostatins. During incubation, samples were collected periodically (6 h, 1, 3, 5, 7, and 10 days) to determine the appropriate incubation time for refolding. Samples were subjected to 10% SDS–PAGE under reduced (+MCE) and non-reduced (–MCE) conditions, and visualized by silver staining (MCE, β-mercaptoethanol). (A) Dimer of unprocessed myostatin (upper arrow) is visible under non-reduced SDS–PAGE. Smaller molecular weight bands indicate misfolded myostatin monomers. Under reduced SDS–PAGE, only a single band of the monomer of unprocessed myostatin is visible (lower arrow). (B) The recombinant mature myostatin did not show the reversible disulfide-linked homodimer formation, as demonstrated by the lack of appearance of the dimer of mature myostatin under non-reduced condition.

well as at least three bands of misfolded monomers between 50 and 37 kDa were observed in non-reduced SDS–PAGE, while a 50 kDa single unprocessed myostatin monomer band (lower arrow) was observed in reduced-SDS–PAGE. The proportion of myostatin dimer increased with the increase in incubation time. Based on the visual evaluation of the thickness of the myostatin dimer band, at least 1 week of incubation in refolding buffer appeared to be required for optimum yield of refolded, unprocessed myostatin. In contrast,

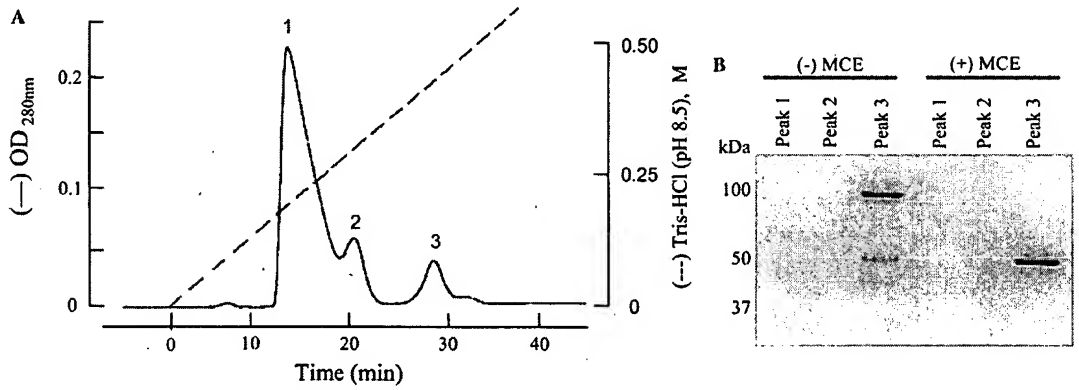

Fig. 3. Purification of refolded, unprocessed myostatin by anion-exchange chromatography. (A) Myostatin solubilized from inclusion bodies was incubated in refolding buffer for 1 week at 4 °C and then dialyzed against 20 mM Tris–HCl buffer (pH 8.5). After dialysis, the solution was applied to an anion-exchange column and eluted with linear gradient Tris–HCl buffer while collecting 2 ml fractions. (B) Fractions containing the peaks observed in the chromatogram were subjected to 10% SDS–PAGE under reduced (+MCE) and non-reduced (–MCE) conditions, and visualized with Coomassie blue staining. MCE, β-mercaptoethanol.

the recombinant mature myostatin did not demonstrate the reversible disulfide-linked dimer formation, indicating that the mature myostatin did not refold properly under the current refolding conditions. Furthermore, centrifugation of the refolding buffer solution containing the recombinant mature myostatin resulted in precipitation of the mature myostatin without any visible protein bands in SDS–PAGE analysis of the supernatant, indicating that the aggregation of the proteins occurred during the refolding period (data not shown). Therefore, no attempt was made to purify refolded, mature myostatin.

Purification of refolded, unprocessed myostatin by anion-exchange chromatography

The result of anion-exchange chromatography of the refolded, unprocessed myostatin solution is shown in Fig. 3A. The three peaks observed in the chromatogram were analyzed by SDS–PAGE (Fig. 3B). Peak 3 eluting at around 0.45 M Tris–HCl contained the unprocessed myostatin, but no protein bands were detectable in peaks 1 and 2. The molecular weight of the unprocessed myostatin in peak 3 was close to 50 kDa under reducing conditions and 100 kDa under non-reducing conditions, indicating a disulfide-linked homodimer formation of the refolded, purified, unprocessed myostatin. Peak 3 also contained small amount of misfolded non-dimer forming myostatin, as demonstrated by the presence of two bands with molecular weights close to 50 kDa under non-reduced SDS–PAGE. The recovery of refolded, unprocessed myostatin after ion-exchange chromatography was about 18% of the inclusion body protein (Table 1).

Purification of refolded, unprocessed myostatin by affinity chromatography

When refolded, unprocessed myostatin was eluted from a nickel affinity column using a stepwise gradient of EDTA, two absorbance peaks were seen (Fig. 4A). When the two peaks and the pass-through solution

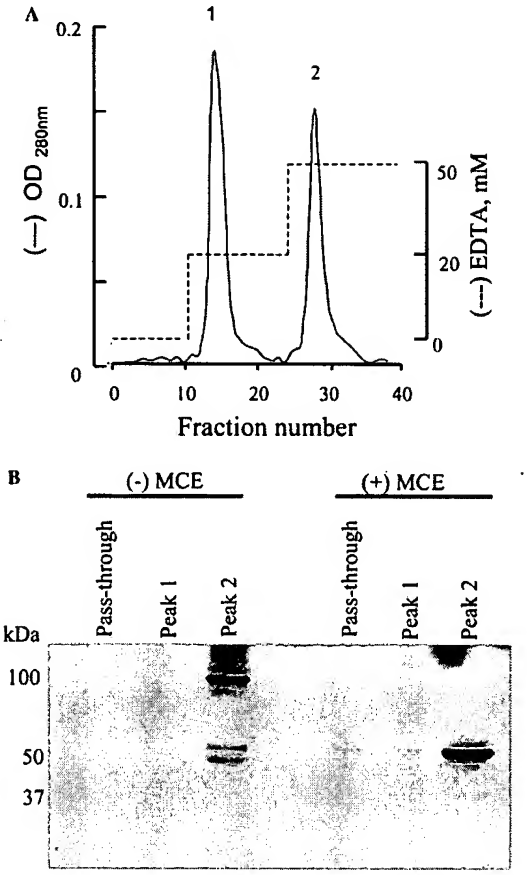

Fig. 4. Purification of refolded, unprocessed myostatin by affinity chromatography. (A) Refolded myostatin solution (250 ml) was loaded into an immobilized nickel affinity column equilibrated with 20 mM Tris–HCl buffer (pH 8.5) containing 0.5 M NaCl. After loading, the column was washed with equilibration buffer and then eluted stepwise with 20 mM EDTA and 50 mM EDTA in equilibration buffer at a flow rate of 1.74 ml/min. The absorbance was monitored at 280 nm and 2 ml fractions were collected. (B) Pass-through solution from sample loading and fractions containing peaks 1 and 2 were subjected to 10% SDS–PAGE under reduced (+MCE) and non-reduced (−MCE) conditions, and visualized with Coomassie blue. MCE, β-mercaptoethanol.

Table 1
Recovery of recombinant porcine myostatin during refolding and purification steps

Purification steps	Recovery of protein (mg)[a]	Yield (%)
Inclusion body protein	1.0	100
Solubilization	0.79	79
Refolding	0.31	31
Ion-exchange or affinity chromatography	0.18	18
Size-exclusion chromatography	0.15	15

[a] Protein concentration was measured by Bradford method (33) using BSA as a standard.

from loading were analyzed by SDS–PAGE, only peak 2 contained the unprocessed myostatin (Fig. 4B). Also apparent in Fig. 4B is that the affinity-purified refolded, unprocessed myostatin formed a monomer under reduced conditions and a dimer under non-reduced conditions, analogous to the refolded myostatin purified by ion-exchange chromatography. Misfolded myostatin also eluted during the affinity purification process, as was evident from the presence of two protein bands with molecular weight close to 50 kDa under non-reduced conditions. Similar to the yield of ion-exchange chromatographic purification, the recovery of

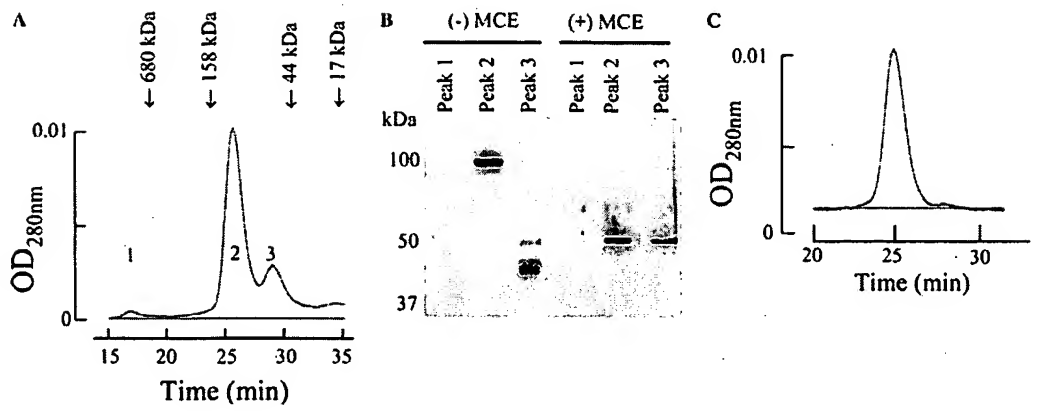

Fig. 5. Size-exclusion gel chromatography of refolded, unprocessed myostatin. (A) Myostatin-containing fractions from ion-exchange chromatography were pooled and subjected to size-exclusion gel chromatography. (B) Peaks were analyzed by 10% SDS–PAGE under reduced (+MCE) and non-reduced (−MCE) conditions, and visualized with silver staining. (C) Peak 2 was reinjected to the size-exclusion gel column to monitor the degradation and purity of the myostatin dimer.

refolded, uprocessed myostatin after affinity chromatography was about 18% of inclusion body protein (Table 1).

Size-exclusion gel chromatography of refolded, unprocessed myostatin

To separate the unprocessed myostatin dimers from misfolded, unprocessed myostatin monomers, myostatin-containing fractions from ion-exchange or affinity chromatography were pooled and subjected to size-exclusion gel chromatography (Fig. 5). As expected, two peaks corresponding to the sizes of unprocessed myostatin dimers (100 kDa) and monomers (50 kDa) were observed (Fig. 5A), and SDS–PAGE analysis of the factions containing the corresponding peaks supported the separation of unprocessed myostatin dimers and misfolded monomers (Fig. 5B). When the purified dimer fractions collected from the size-exclusion gel chromatography were subjected to another round of size-exclusion gel chromatography, a single peak corresponding to the dimer size reappeared (Fig. 5C), indicating no formation of aggregates after purification. The protein assays showed that unprocessed myostatin dimers accounted for about 85% of total protein, thus the yield of purified, refolded myostatin after size-exclusion chromatography was about 15% of inclusion body proteins (Table 1).

Isoelectro-focusing and native gel analysis of refolded, unprocessed myostatin

Isoelectro-focusing and native gel electrophoresis of the purified, unprocessed myostatin are shown in Fig. 6. The purified myostatin appeared as a sharp single band in both electrophoretic analyses, indicating homogeneity of the purified, refolded, unprocessed myostatin with respect to charge and size. The p*I* of the refolded myostatin was around pH 7.0, which is not far from the theoretical p*I* of 6.3 of the porcine unprocessed myostatin estimated by the ProtParam tool available from the Swiss Institute of Bioinformatics [18].

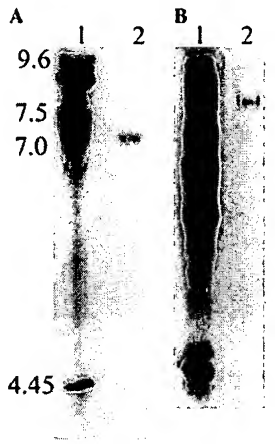

Fig. 6. Isoelectro-focusing and native gel analysis of refolded, unprocessed myostatin. (A) Myostatin-containing fractions from ion-exchange and affinity chromatography were pooled and subjected to isoelectro-focusing (IEF) with the pH gradient from 4.45 to 9.6 in order to determine the p*I* and homogeneity of the purified myostatin. After fixing, the gel was stained with Coomassie blue. Lane 1, protein standard for IEF; lane 2, purified myostatin. (B) To determine the homogeneity of the purified myostain, myostatin-containing fractions from ion-exchange and affinity chromatography were pooled and subjected to 10% native PAGE, and visualized with Coomassie blue staining. Lane 1, standard for native PAGE; Lane 2, refolded myostatin.

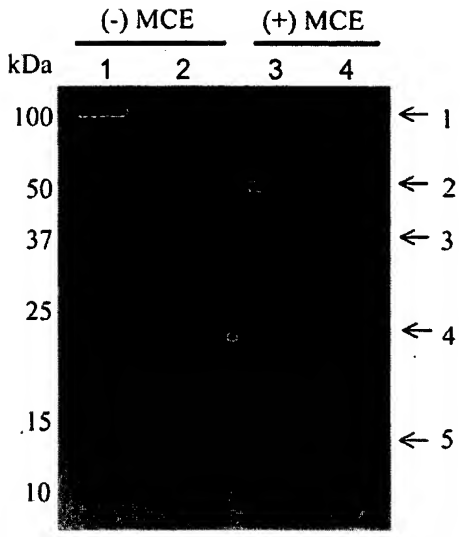

Fig. 7. Furin proteolysis of the refolded, unprocessed myostatin. (A) The refolded myostatin purified by size-exclusion chromatography was incubated with furin overnight at 30 °C in reacting buffer. The reaction mixture was subjected to 15% SDS–PAGE under reduced (+MCE) and non-reduced (−MCE) conditions, and visualized with Coomassie blue staining. Lanes 1 and 3 contained refolded myostatin not reacted with furin, and lanes 2 and 4 contained refolded myostatin reacted with furin. Furin reaction yielded prodomain (arrow 3) and mature form of myostatin (arrow 5) under reduced condition. Mature myostatin formed a dimer under non-reducing conditions (arrow 4), but the prodomain did not. Arrows 1, 2, 3, 4, and 5 indicate unprocessed myostatin dimer, unprocessed myostatin monomer, prodomain monomer, mature myostatin dimer, and mature myostatin monomer, respectively. MCE, β-mercaptoethanol.

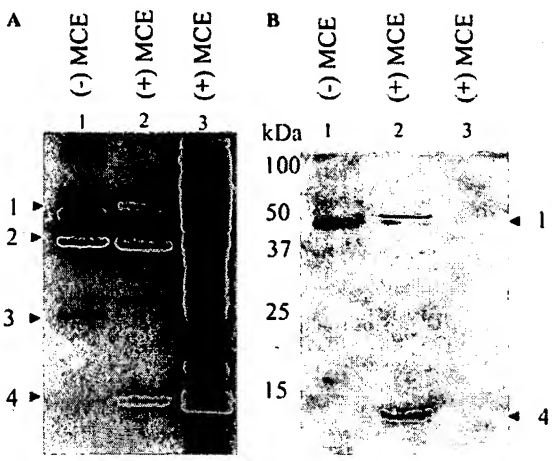

Fig. 8. Western blot analysis of furin-digested refolded, unprocessed myostatin. (A) Furin digested mixture was subjected to 15% SDS–PAGE and visualized with Coomasie blue staining. Lane 1: furin-digested myostatin (−MCE); lane 2; furin-digested myostatin (+MCE); and lane 3, liver homogenate (+MCE). (B) Myostatins fractionated by SDS–PAGE were electrophoretically transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with polyclonal anti-myostatin antibodies raised against a peptide region of mature myostatin and then subsequently incubated with secondary antibodies conjugated with alkaline phosphatase, followed by color development with the BCIP/NBT substrate. Arrowheads 1, 2, 3, and 4 indicate unprocessed myostatin monomer, prodomain, mature myostatin dimer, and mature myostatin monomer, respectively. MCE, β-mercaptoethanol.

Furin proteolysis of refolded, unprocessed myostatin

The mature (active) form of myostatin is formed upon removal of the N-terminal prodomain from unprocessed myostatin by proteolysis at the paired basic (Arg–Lys–Arg–Arg) residue [7]. The paired basic residue is preferably recognized by furin, an endopeptidase involved in the conversion of a wide variety of precursor proteins into their mature forms [19]. Since our recombinant myostatin was expressed as an unprocessed form carrying the paired basic site, the refolded myostatin was reacted with furin to determine the proteolytic processing of the refolded, unprocessed myostatin. Furin proteolysis (Fig. 7A) yielded two proteins with molecular weights of approximately 37 kDa (arrow 3) and 13 kDa (arrow 5) under reduced SDS–PAGE, corresponding to the N-terminal prodomain and the C-terminal mature form of myostatin, respectively. Under non-reduced SDS–PAGE, the 37 kDa prodomain remained as a 37 kDa band, but the 13 kDa band disappeared and, a 24 kDa band (arrow 4) appeared. The result demonstrates the disulfide-linked homodimer formation of the 13 kDa mature myostatin and no disulfide-linked interaction between the prodomain and mature myostatin or between prodomain monomers.

Western blot analysis of refolded myostatin

Using a commercially available polyclonal antibody that was generated against a synthetic peptide from the mature form, binding characteristics of the furin-digested products of unprocessed myostatin were analyzed by Western blotting (Fig. 8). As expected, the 37 kDa prodomain (arrow 2) had no affinity to the antibody, while the unprocessed myostatin monomer (arrow 1) and the 13 kDa mature myostatin (arrow 4) did show affinity to the antibody. However, the 24 kDa dimer of mature myostatin (arrow 3) did not show any affinity, suggesting that the dimer formation of mature myostatin prevented the exposure of epitopes recognized by the antibody.

Discussion

The mature form of myostatin as well as the precursor form of myostatin have been reported to form disulfide-linked homodimers, like many other members of the TGF-β superfamily [1,7]. In a study with Chinese

hamster ovary (CHO) cells carrying copies of a murine myostatin expression construct, it was reported that two myostatin-immunoreactive proteins were present under reducing condition: 52 and 15 kDa, representing monomers of the unprocessed precursor and mature forms of myostatin, respectively [7]. Under non-reducing condition, two proteins of 101 and 25 kDa were detected, representing dimeric forms of unprocessed and mature myostatin, respectively. Dimer formation of the mature form of myostatin has also been demonstrated in several other studies in vitro [1,7,8]. Therefore, the reversible disulfide-linkage formation was used as an index for proper folding during the refolding process employed in this study.

While the unprocessed porcine myostatin demonstrated a reversible disulfide bond formation after refolding, characteristic for native forms of myostatin, the mature recombinant myostatin did not. Rather, the mature form aggregated without the demonstration of reversible disulfide-linked homodimer formation in the refolding condition used in this study. These results demonstrate that the myostatin prodomain facilitates the proper folding of mature myostatin allowing dimer to form. Various attempts in this study with different combinations of refolding parameters were not successful in refolding the mature myostatin, emphasizing the role of the myostatin prodomain in proper folding of mysotatin at least in vitro. The requirement of the prodomain for proper folding and secretion of mature protein has been demonstrated in many members of the TGF-β superfamily that contain the characteristic cystine knot [11,12]. Similar to our results, when only the mature sequences of activin A and TGF-β1 were expressed in eukaryotic cells, disulfide-linked aggregates of activin A and TGF-β1 were formed in the transfected cells without secretion of those proteins, indicating an intracellular role of the prodomains in the formation of correctly located disulfide bridges in the mature form of those proteins [11]. While the current results suggest that the myostatin prodomain plays a role in proper folding of myostatin in vitro, the intracellular role in folding in vivo remains yet to be examined since most studies that expressed myostatin in eukaryotic cells employed expression vectors containing the unprocessed myostatin sequence [1,7,10]. According to the study by Gray and Masson [11], the *trans* presence of prodomain achieved by co-transfection was effective in the secretion of the homodimer of TGF-β1 and activin A, even though the *cis* presence of prodomain was 10 times more efficient in secreting properly folded mature TGF-β1 and activin A in eukaryotic cell expression systems. In this regard, it will be interesting to examine whether the *trans* presence of myostatin prodomin aids the refolding of mature myostatin in vitro and in vivo as well. If proved effective, the *trans* expression of myostatin prodomain will probably improve the efficiency of production of mature myostatin by eliminating a step involved in removing the prodomain after the folding of unprocessed myostatin.

The refolded, unprocessed myostatin was able to be purified using a combination of size-exclusion chromatography and either ion-exchange chromatography or affinity chromatography. The purified, unprocessed myostatin was homogeneous as demonstrated by a sharp single band in isoelectric-focusing and native gel electrophoresis. After reaction with furin, an endopeptidase that separates the prodomain from mature myostatin, the purified unprocessed myostatin yielded two proteins of 37 and 13 kDa, the correct size for prodomain and mature myostatin, respectively. Consistent with the known characteristic of homodimer formation of mature myostatin, the 13 kDa fragment formed disulfide-linked homodimer under non-reduced condition. Unlike the TGF-β prodomains that form disulfide-linked dimers [20–22], the myostatin prodomain has been reported not to form disulfide-linked dimers [7,8]. Our data also show that the 37 kDa prodomain cleaved from the unprocessed myostatin by furin did not form disulfide-linked dimers. The prodomains of some other members of TGF-β superfamily, such as BMP-2 and -7, are also known not to form disulfide-linked dimers [12,23].

It is interesting to note that the dimer of mature myostatin was not recognized by the commercial polyclonal anti-myostatin antibody while the myostatin monomer was recognized in Western blot analysis. Considering that the antibody was raised against a peptide from the mature myostatin sequence, this difference in binding to the antibody between the monomer and dimer forms indicates that the peptide used as an antigen in antibody production was probably from a region of the protein that is hidden during dimer formation and thus not accessible to the antibody. Similar results were found when anti-TGF-β1 antibodies raised against peptides corresponding to various regions of TGF-β1 were tested for their binding characteristics to TGF-β1 [24]. The amino-terminal region appeared to be inaccessible by antibodies when the TGF-β1 was in the dimeric conformation. Crystal structure studies of TGF-β2 revealed that dimer formation buries a large surface area of each monomer [25], indicating that when antibody is raised against peptide regions of a protein, the choice of peptide region is an important factor in successful production of antibody with high affinity for native proteins. Unlike the anti-myostatin antibody raised against a peptide region of mature myostatin, it was reported that anti-myostatin antibodies raised against the full sequence of the mature form recognized both the dimer and monomer forms of myostatin [1,7]. The above results indicate that careful validation of binding characteristics of anti-myostatin antibodies is essential in using anti-myostatin antibodies to determine

the level of tissue or serum myostatin in various physiological states.

In conclusion, we demonstrate that highly efficient refolding in vitro is possible for the unprocessed porcine myostatin, but not for the mature form of myostatin expressed in *E. coli* as inclusion bodies, and that the myostatin prodomain facilitates the folding of mature myostatin.

Acknowledgments

This study was supported by USDA-TSTAR Grant (99-34135-8109) and USDA NRICGP Grant (00-35208-9418).

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